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Discussion and future perspectives

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Genotype-phenotype correlation of mutations in the genes encoding the basal and suprabasal keratins

Epidermolysis bullosa simplex (EBS) was the first hereditary skin blistering disorder of which the etiology was established in the early 1990s.[1-3] EBS was also the first genetic disease caused by mutations in genes encoding intermediate filament (IF) proteins, namely the basal epidermal keratins K5 and K14. The next hereditary skin disorder in which keratin defects were found to underlie the disease was epidermolytic ichthyosis (EI) with mutations in the genes *KRT1* and *KRT10* encoding the suprabasal keratins K1 and K10, in 1992.[4-6] Since then a wealth of studies, involving these disorders and their causal genes and proteins, have emerged. At present 193 different mutations have been reported in the genes *KRT5* and *KRT14* encoding the keratins K5 and K14, of which 171 mutations are heterozygous/dominant and associated with one of the major EBS subtypes: EBS localized (EBS-loc), EBS generalized non-Dowling Meara (EBS-gen), and EBS Dowling Meara (EBS-DM) (www.interfil.org).[7] A total of 115 mutations has been reported for K1 and K10 at present.[7] The large majority of the mutations are dominant missense or small in-frame deletions or insertions. From all these reported mutations and their associated phenotypes, it appeared that correlations exist between the location and nature of the mutations, the extent of distortion of the keratin cytoskeleton integrity, and eventually the severity of the clinical features. Mutations affecting the residues in the helix boundary motifs of the rod domains of K5 and K14 are predominantly associated with the most severe EBS-DM phenotype, whereas mutations located in the more central parts of the rod domain and the linker domains cause EBS-loc and EBS-gen. Mutations in the variable head and tail domains of K5 and K14 cause atypical phenotypes, of which some are not even associated with skin fragility. In EI, the most severely affected patients carry mutations in the helix boundary motifs of K1 and K10, while milder cases carry mutations in the more central parts of the rod. The few head and tail domain mutations described for K1 cause atypical phenotypes, similar to what is observed for the basal keratins. In K10, no head or tail domain mutations have been reported at all. In general, hardly any mutations haven been reported that were located outside the rod domains of K1 and K10.

Pathogenicity of L12 linker domain mutations in K1 and K10

Mutations in the linker domains of K5 and K14 cause mild EBS, whereas for the suprabasal keratins K1 and K10 only one linker domain mutation (in the K1 L12 domain) had been reported.[8] These data suggest that mutations affecting the linker domains of K1 and K10: 1. do not cause a phenotype; or 2. cause such mild phenotypes that no medical attention is sought; or 3. are not recognized because of their unusual appearance. In **chapter 4** the second linker domain mutation in one of the suprabasal keratins is described: a missense mutation also in the L12 domain of K1. This mutation was found in affected persons of three families, and was associated with an atypical, mild phenotype with prominent palmoplantar keratoderma and mild skin

fragility, whereas the, for EI characteristic, ichthyotic scaling and neonatal erythroderma were lacking. Of note, the clinical features resembled the phenotype of the patients with the one other, previously reported K1 L12 domain mutation.[8] With transfection studies we have shown that both the novel and the previously reported K1 L12 mutation cause keratin aggregations *in vitro*. This effect was aggravated upon application of a hypo-osmotic stress stimulus to the cells. In general, the functions of the linker domains of IFs are not well known. The results of the transfection studies performed in **chapter 4** indicate that the L12 domain is involved in normal keratin assembly and stress resistance. The K1 L12 domain mutations cause mild phenotypes that might be missed because of the lack of resemblance to classical EI. The observation of unexplainable palmoplantar keratoderma in combination with mild skin fragility, blistering and/or peeling-skin like features should raise the suspicion of EI due to K1 linker domain mutations, especially when suprabasal epidermolysis and/or keratin aggregation is observed in ultrastructural analysis of patient's skin. Notably, in view of this mild blistering phenotype, EI could also be considered as a form of suprabasal EBS, although this was not acknowledged in the latest EB consensus, mainly because EI is generally thought of as an ichthyosiform, scaling disorder.

Recently, we have discovered a novel, dominantly inherited missense mutation in the K10 L12 domain in a family with an even milder, more acral-peeling-skin-syndrome-like phenotype (unpublished). In general, mutations in the type II keratin K1 are associated with more severe phenotypes than mutations in K10 (type I). Moreover, recessive loss-of-protein expression mutations have been reported for K10, but not for K1. Similarly, in basal keratins only recessive loss-of-protein mutations have been observed for the type I keratin K14, but not for the type II K5. Furthermore, K5-knockout mice show a more severe and earlier lethal phenotype then the K14-knockout mice.[9] All these findings most likely reflect the functional redundancy of keratins. K14 loss can be (partly) compensated for by another basal keratin, K15, and upregulation of the wound-repair keratin K16. Furthermore, K10 mutations can be (partly) compensated for by expression of an additional type I keratin (K9) in palmoplantar skin.[10] On the other hand, patients with K9 mutations have palmoplantar keratoderma, so apparently K10 cannot compensate for K9 mutations. The type I keratins K5 and K1 have no alternative heterodimer partners, and therefore alterations in these proteins may cause more severe phenotypes. Alternatively, functional properties of type I and type II proteins differ. Of note, the phenomenon of functional redundancy of keratins is interesting in the light of therapeutic options for hereditary keratin disorders, as upregulation of other keratins may reduce the detrimental effect of the mutated keratin.

Additional transfection studies will reveal whether the novel K10 L12 domain mutation has the same detrimental effect on the keratin cytoskeleton as the K1 L12 mutations *in vitro*, or perhaps has no notable effect and therefore causes such a mild phenotype. It would be interesting to perform transfections studies with the both the K1 and K10 linker domain mutations, and with helix boundary motif-affecting mutations as well. By comparing the effects

of each these mutations on the cytoskeleton, in the unstressed and the stressed state, a better understanding of the roles of the linker domains in keratin assembly and stress resistance, and the phenotypes they inflict when altered, can be obtained.

Genotype-phenotype correlations in the basal EBS population from the Netherlands

The basal EBS population that visited the Center for Blistering Disease in Groningen (the Netherlands) in the last decades is described in **chapter 2**. This is the largest basal EBS cohort reported yet, consisting of 65 unrelated probands. One of the strengths of this study is the confirmation of the level of skin fragility in skin biopsies of all patients. The findings reported in **chapter 2** confirm the general EBS genotype-phenotype correlation mentioned in the previous paragraph. However, from our findings it also appears that, although there is a general concordance between mutation and phenotype, eventually the combination of the position of the mutations within the protein, the position within the protein subdomain structure (i.e. the heptad of the α-helix), the nature of the mutation and additional environmental factors and unknown genetic and epigenetic factors, determine the clinical outcome. This is exemplified by the observed intrafamilial phenotypic variation, the improvement that often appears after puberty, the influence of environmental temperature and humidity, and reports of improvement of blistering during feverish illness. In addition, mutations that affect adjacent residues may result in different phenotypes, and mutations affecting the same residue, although with a different substitution, result in different phenotypes. Most striking is the intrafamilial clinical variation and phenotypic variation between individuals with identical mutations.

Recent studies involve the additional factors influencing phenotypic outcome in hereditary blistering diseases. One study indicated that polymorphisms the matrix metalloproteinase 1 (MMP-1) enzyme, that has type VII collagen as a substrate, influence the degradation activity of this enzyme and modify the phenotype in recessive dystrophic EB (RDEB) patients with mutations in the type VII collagen gene (*COL7A1*).[11] In addition, a second study detected a possible association of the polymorphisms in the MMP-1 gene promoter with early-onset squamous cell carcinoma in RDEB patients.[12] However, in another study in large cohort of European DEB patients no significant correlation between MMP-1 promoter polymorphisms and severity of either dominant or recessive DEB phenotypes, or squamous cell carcinoma development, was found, although a (non-significant) tendency was observed.[13] These data suggest that MMP-1 polymorphisms are not the sole modifiers of DEB disease severity. The discovery of modifier genes and perhaps epigenetic phenomena might have implications for establishing a prognosis and for future therapeutic options. Such modifiers have not been reported for EBS (yet). Modifier gene candidates could be genes coding for proteins that directly interact with K5 and K14, such as desmosomal and hemidesmosomal proteins. But also variations in proteins influencing the overall skin condition, like for example filaggrin (gene: *FLG*), which is associated with ichthyosis vulgaris [MIM #146700] and atopic disease, might have an effect on disease severity. Other possible disease modifiers in EBS might be genetic variations

in genes encoding proteins that are involved in post-translational modifications of keratins, such as phosphorylation, transglutamination, and glycosylation. These possibilities remain to be investigated.

Recently, more attention is paid to the role of inflammation as a modifier in EBS. Several observations point to this association. The clinical EBS migratory (EBS-migr) subtype shows a migrating belt-like erythema with vesicles on the margin (circinate), and also in EBS-DM the blisters have a grouped and/or circinary pattern on erythematous skin, rendering it an inflammatory aspect.[14] Similarly, patients with cyclic-ichthyosis-epidermolytic-hyperkeratosis (CIEH [MIM #607602]) caused by mutations in the suprabasal keratins K1 or K10, show dramatic episodic flares of multiple annular, polycyclic erythematous plaques with scaling that lasts for weeks to months and then disappears again.[15, 16] Another interesting phenomenon is the notion of EBS patients that rise of body temperature by upcoming illness like the flu reduced their blister rate, as if the basal cell keratins are stabilized by internal warmth, or alternatively the elicited immune reaction against a (viral) infection with production of cytokines might have its effects on keratin stability and the formation/breakdown of keratin aggregates. Furthermore, outbreaks of severe skin blistering in EBS can be reduced by the use of topical and systemic corticosteroids (personal observation). Upregulation of inflammatory cytokines was shown in K5 and K14 knockout mice, and in skin samples of EBS patients carrying mutations in K5 and K14.[17-19] Studies on keratinocytes of patients with Naegeli-Franceschetti-Jadassohn syndrome due to heterozygous nonsense/frameshift mutations in the first exon of *KRT14*, indicated increased susceptibility for TNF-α-induced apoptosis.[19] In addition, transfection of various *KRT14* mutations into HaCaT-cells resulted in increased TNF-α secretion and increased susceptibility to TNF-α-induced apoptosis, which was reversible upon treatment with anti-TNF-α antibodies.[20] Of note, type I IFs, including K14, sequester the apoptotic-adaptor-TNF-receptor-associated-death-domain (TRADD) in the cytoplasm, thereby regulating apoptosis.[20, 21] In contrast, K5 -/- mice did not show TNF-α upregulation, but instead higher levels of interleukins and other cytokines were detected.[17, 18] Interestingly, the lifespan of the K5 -/- mice could be extended by treatment with the anti-inflammatory drug doxycyclin. In K14 -/- mice another anti-inflammatory molecule, sulphoraphane, which is a substance of broccoli, was shown to prolong survival as well.[22] The mode of action of sulphoraphane partly involves upregulation of keratins K16 and K17 as well. It could be hypothesized that both K5 and K14 have anti-inflammatory capacity, but that the mode of action differs. However, the proinflammatory cytokines may also result from cell lysis in itself and thus be a secondary phenomenon. Whether the in inflammation results from release of cytokines upon cell lysis, or reflects the impaired inflammation-suppressing capacity of K5/K14 due to the head/tail domain mutation remains to be investigated. In the mean time, anti-inflammatory substances and medications, like sulphoraphane, doxycyclin and perhaps anti-TNF-α medications, might provide blister-frequency-reducing treatments in the near future. The search for other agents that upregulate the wound-repair keratins K6, K16 and K17 may provide treatment options in the future as well. In general, the apparent functional redundancy of the

keratin protein family could be further exploited. For example, upregulation of the other basally expressed keratin, K15, could proof to be a therapeutic option for EBS.

K5 and K14 head and tail domain mutations: unusual phenotypes

In our EBS population presented in **chapter 2** three mutations (p.Pro25Leu, c.1635delG, c.1649delG) affect the head and tail domains of K5. These mutations have been previously reported and similar as in our study two of these mutations, p.Pro25Leu and c.1649delG, were also associated with atypical phenotypes with pigmentation abnormalities and/or blistering with an inflammatory aspect. The p.Pro25Leu mutation is well known for its involvement in mild skin fragility and mottled pigmentation: EBS-MP. Moreover, this mutation has exclusively been associated with this phenotype in different populations.[23-31] The C-terminal K5 mutation c.1649delG causes a frameshift and subsequently a delayed termination codon leading to a longer K5 tail.[14, 32] This mutation has been associated with EBS-MP as well, and additionally an inflammatory-like EBS phenotype with migratory circinate erythema and vesicles with skin pigmentation abnormalities. The other K5 C-terminal mutation in our study, c.1635delG, was associated with an EBS-gen phenotype with additional pigmentation disturbances. In a previous study a patient carrying this same mutation showed an EBS-loc phenotype without pigmentation abnormalities being mentioned.[33] The fourth mutation affecting one of the variable domains of K5/K14 is the novel mutation *KRT14*:c.1240-1249del10. This mutation is located in the last exon of *KRT14* and predicted to cause a frameshift and a premature termination codon 25 amino acids downstream, resulting in an aberrant K14 HTM and truncation of the K14 tail. Similar to the K5 tail mutations, this mutation was associated with an inflammatory blistering phenotype. However, the patient at present is only two years old and the further course will have to reveal whether pigmentation disturbances become part of the phenotype as well. The findings in patients with K5 and K14 head and tail domain mutations implicate that these domains have a function in skin pigmentation regulation and inflammation (also discussed in the previous paragraph). Findings in other hereditary skin disorders associated with mutations in the K5 and K14 head and tail domains support this idea. These genodermatoses are Dowling-Degos Disease (DDD), Naegeli-Franceschetti-Jadassohn syndrome (NFJS) and Dermatopathia Pigmentosa Reticularis (DPR). DDD is an autosomal dominant pigmentary disorder characterized by postpubertal progressive reticulate hyperpigmentation and small, hyperkeratotic dark brown papules mainly affecting the flexures and great body folds. In DDD, filliform extensions of basal keratinocytes into the dermis, abnormal intracellular distribution of melanosomes in basal keratinocytes, and an irregular organisation of melanin pigments within melanosomes, has been described.[34] DDD is caused by heterozygous *KRT5* nonsense and frameshift mutations in the K5 head domain. NFJS and DPR are genetic skin disorders which are also characterized by pigmentation disturbances, and are caused by heterozygous nonsense and frameshift mutations in the K14 head domain. NFJS is clinically characterized by absence of dermatoglyphs and reticulate hyperpigmentation that tends to resolve with age, palmoplantar keratoderma, decreased sweating, and sometimes

dental abnormalities. DPR shares features with NFJS but is distinguished by a lifelong presence of skin hyperpigmentation, partial alopecia, and absence of dental abnormalities. DDD, NFJS, and DPR all result from nonsense or frameshift mutations that are predicted to cause loss of protein expression from the affected allele, although RNA and protein studies are not unequivocal. Of note, as unaffected carriers of recessive KRT14 loss-of-protein expression mutations do not show a phenotype, it is yet unclear if haploinsufficiency is underlying NFJS and DPR. It is also possible that (small amounts) of truncated K5 and K14 proteins, or K5 and K14 proteins with defects in their head domain due to in-frame changes, result from these mutations. Furthermore, the phenotypes of NFJS, DPR, and DDD do not include skin fragility, implying that mutations causing K5/K14 haploinsufficiency, truncated K5/K14 proteins, or altered K5/K14 head domains, do not necessarily lead to keratin cytoskeleton fragility.

In general, the unusual phenotypes associated with mutations in the head and tail domains of K5 and K14, but also of the suprabasal keratin K1, point to functions of keratins beyond cell stability and stress-resistance. The process of keratin assembly and the role of the conserved rod domain have been subject of many studies. However, less is known about the functions of the variable domains of keratins. The head and tail domains, as indicated by their variability, likely give each keratin its specific functional properties in the cells in which they are expressed. For example, post-translational modification processes of IFs, such as phosphorylation, transamination and glycosylation, take place via the head and tail domains.[35, 36] Specific mutations in these domains would be predicted to interfere with these processes.

Concerning the pigmentation abnormalities observed in patients with mutations affecting the K5/K14 head and tail domains several interesting observations can be noted. The K5 head domain has been observed to bind proteins which are involved in intracellular organelle transport and localisation, like dyneins and myosin V. Dyneins are engaged in melanosome localisation and retrograde transport of IF proteins[37, 38] and myosin V has been shown to interact with IFs. Moreover, mutations affecting the myosin V-gene are also associated with a pigmentary disorder, Griscelli syndrome [MIM#214450].[39-42] It can be hypothesized that mutations in K5 (and/or K14) head disrupting the interaction with these proteins lead to altered melanosome distribution in the cell, such as observed in DDD, and lead to clinically observable pigmentation abnormalities.[37, 38]

Binding sites for inflammatory cytokines have been reported for the head and tail-domains of IFs as well. Possibly, mutations affecting these binding could induce phenotypes with an inflammatory appearance (see also previous paragraph). Furthermore, the non-helical head domain of type II keratins, including K1 and K5, is important for the anchorage to cell-cell contacts, by means of binding to desmoplakin (DP) in the desmosomal inner plaque.[43-45] K14 does not have this property. The interaction of the basal keratins to the hemidesmosomal plaque protein BP230 and plectin is likely to take place by means of the K5 head as well, considering the homology between plectin and DP. However, this has not been investigated yet. The careful clinical and skin tissue morphological evaluation of patients with mutations in the head and

tail domains of the epidermal keratins, *in vitro* studies of behaviour of keratinocytes containing these mutations, and studies investigating the protein interactions of the head and tail domains of keratins, and the dose effects of reduced keratin expression, may provide clues about the diverse interactions and functions of these domains in inflammation, intracellular organelle transport and signalling cascades. Furthermore, elucidation of the exact effects of the *KRT5* and *KRT14* nonsense and frameshift mutations on the K5 and K14 proteins may give a better understanding into the genotype-phenotype correlation and are important for studies aiming at knocking down the dominant, mutation-carrying allele as a treatment for EBS.

Plectin mutations in non-syndromic basal EBS

Notable observations in the EBS population presented in **chapter 2** were the high number of *de novo* mutations and the failure to detect mutations in K5 or K14 in 25% of the cases. This latter observation was also reported for the EBS population in the United Kingdom (22%).[46] These data suggest genetic heterogeneity underlying EBS. Single reports of mutations in hemidesmosomal proteins, like plectin, integrin β4, and BP180, causing non-syndromic EBS, have appeared.[47-50] One of these, affecting the plakin protein plectin, was particularly interesting, as this was an autosomal dominant mutation.[50] The heterozygous missense mutation, *PLEC1*:p.Arg2000Trp, in the α-helical rod domain of plectin was found in affected persons in a large Norwegian and an unrelated German family with skin fragility with an intraepidermal plane of cleavage through the basal keratinocytes just above the hemidesmosomes.[50, 51] The phenotype was named EBS-Ogna.[51] In **chapter 3** we have shown that heterozygous plectin missense mutations underlie basal EBS in four probands of our own basal EBS cohort in which mutations in K5 and K14 had been carefully excluded. The plectin missense mutation that was detected in the original Norwegian and German EBS-Ogna patients, p.Arg2000Trp, was also found in two of these four probands. The clinical features of the Dutch EBS probands with mutation p.Arg2000Trp, were strikingly similar to those of the original Norwegian EBS-Ogna patients, as were also the ultrastructural and immunofluorescence findings.[50-52] The higher frequency of this mutation at residue p.Arg2000 might be due to deamination of a methylated cytosine in the context of a CpG dinucleotide, the most frequent mutation event in the human genome.[53] Although the numbers are low, one might speculate that this is a mutation hotspot. Additional studies in other non-K5/K14 EBS populations are necessary to confirm this. The other plectin missense mutations found in the study presented in **chapter 3** were located in the C-terminal plakin repeat domains (PRDs). As mentioned, these domains are thought to be involved in anchorage of IFs, in addition to several other binding partners. How the different missense mutations affect the function of the plectin rod and the C-terminal PRDs is yet speculation (see also **chapter 3**). Proving pathogenicity of missense mutations is difficult, especially in large proteins, such as plectin, that have many isoforms and multiple binding partners. However, the mutations were not found in >150 matched control DNA samples and fully segregated with the phenotype within

the families. These findings are in strong support of the pathogenicity of these substitutions. Transfection studies could give further insight, but this was outside the scope of the study. As mentioned above, *PLEC1* mutation analysis in EBS patients with wild-type *KRT5* and *KRT14* genes in other populations can give additional insight.

A significant difference was observed between the patients with the rod-domain mutation p.Arg2000Trp and the patients with the C-terminal mutations upon immunofluorescence staining of patients' skin samples with anti-plectin antibodies 10F6 and 5B3. Immunofluorescence stainings with these antibodies were markedly reduced to absent in the basal epidermal layer and along the epidermal basement membrane zone (BMZ) in the patients with mutation p.Arg2000Trp, while staining was near normal in the patients with the C-terminal mutations. This suggests that the rod domain missense mutation either changes the epitope for these antibodies, or, alternatively, causes degradation of the isoforms containing these epitopes in the basal layer. In general, the impressive variability of staining patterns of different anti-plectin antibodies is puzzling (figure 1), as is also the variety of tissues affected (in different combinations) by the multiple plectin mutations previously described (figure 2, and table 1 at the end of this chapter).

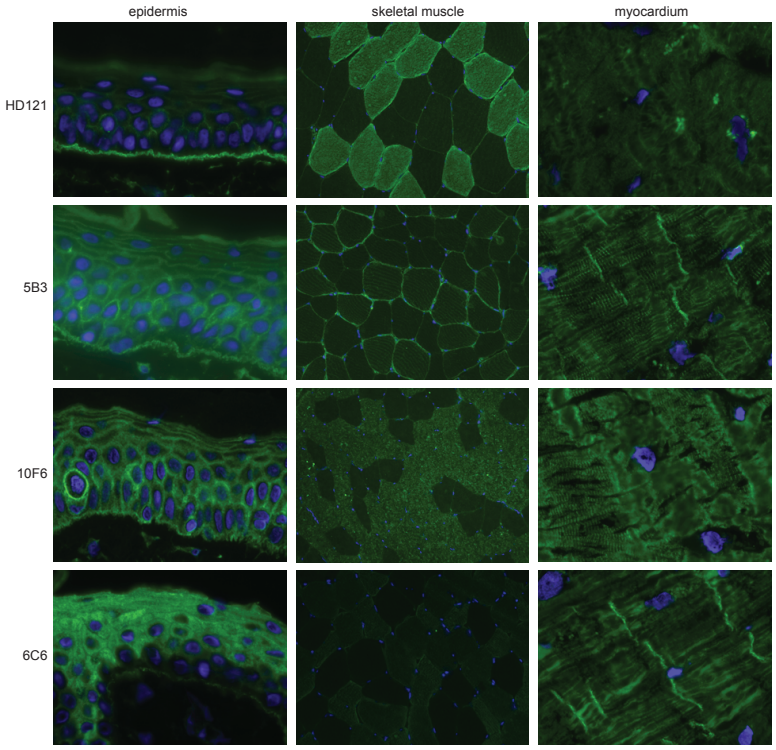

Figure 1. The various immunofluorescence staining patterns of different anti-plectin antibodies in normal skin, skeletal muscle and cardiac muscle samples.

Many antibodies have been mapped, mostly to the plectin rod domain. The fact that many different plectin isoforms exist (eight different human plectin isoforms can be found in GenBank), suggests that these antibodies may recognise different plectin isoforms. However, all plectin isoforms described in GenBank are similar in their rod and C-terminus and only differ in the first 10-300 aminoacids (although there is also evidence for the existence of an additional, rodless plectin isoform, generated by out-splicing of exon 31[54]). Therefore the different staining patterns of antibodies 10F6, 5B3, and HD121, which all have epitopes mapped to the plectin rod, can not be explained based on the currently known isoforms of plectin.[55, 56] Possible reasons for the markedly different staining patterns of these anti-plectin rod antibodies may be: 1) there are (yet undiscovered) plectin isoforms differing in their rod domains; 2) the anti-plectin antibodies have conformation-dependent epitopes that differ per plectin localisation within the different parts of the skin, and/or even within the cell; 3) the epitopes are bound *in vivo* by their binding-partner proteins in a tissue-, cell- and even structure-specific manner and therefore at certain positions particular epitopes are blocked for the antibodies. Recently, antibodies specific against the N- and C-terminus of plectin, and plectin isoform-specific antibodies have been reported.[57-59] Additional immuno-EM antigen mapping using these different antibodies in the different tissues, i.e. skin, skeletal muscle, heart, may give insight in the specific expression of the multiple plectin isoforms, and perhaps indicate the existence of yet unidentified isoforms. This may then help in further elucidating the genotype-phenotype correlations of *PLEC1* mutations.

When taking all previously reported *PLEC1* mutations into account, at least one tendency seems to be present: homozygous and compound heterozygous loss-of-function mutations affecting both the full-length and the rodless plectin isoforms (nonsense or frameshift mutations located outside exon 31), cause severe and early lethal phenotypes in humans (see figure 2, and table 1 at the end of this chapter).[54, 60] Furthermore, mice that are knockout for both the rodless and the full-length plectin isoforms also show early postnatal lethality with severe mucocutaneous fragility.[61] In contrast, patients with EBS-MD overall survive much longer and have less severe skin blistering. These patients usually have loss-of-function mutations located within exon 31 and therefore still have functioning rodless plectin. It is thought that this rodless isoform can partly compensate for loss of full length plectin and hereby protects these patients from an early lethal phenotype. As mentioned above, insight in the precise localisation and function of the different plectin isoforms within tissues may give further insight in the phenotype-genotype correlation of *PLEC1* mutations, and may possibly lead to linkage of molecularly unsolved genetic diseases to plectin mutations.

To summarize, in **chapter 3** missense mutations in the gene *PLEC1* encoding plectin were shown to underlie EBS in four of 16 EBS cases (25%), i.e. four out of a total of 65 EBS families (6%). These findings implicate that plectin pathology is not as rare in non-syndromic EBS as was initially thought, and that in case of EBS with negative *KRT5* and *KRT14* mutation screening, *PLEC1* is the next candidate gene. Additional clinical features that may indicate underlying plectin

pathology are onychogryphosis of the big toenails, and development of small blood blebs on the hands upon mild mechanical trauma. In skin biopsies a pseudojunctional cleavage and absent basal layer staining with antibody 10F6, which is commercially available, are additional clues.

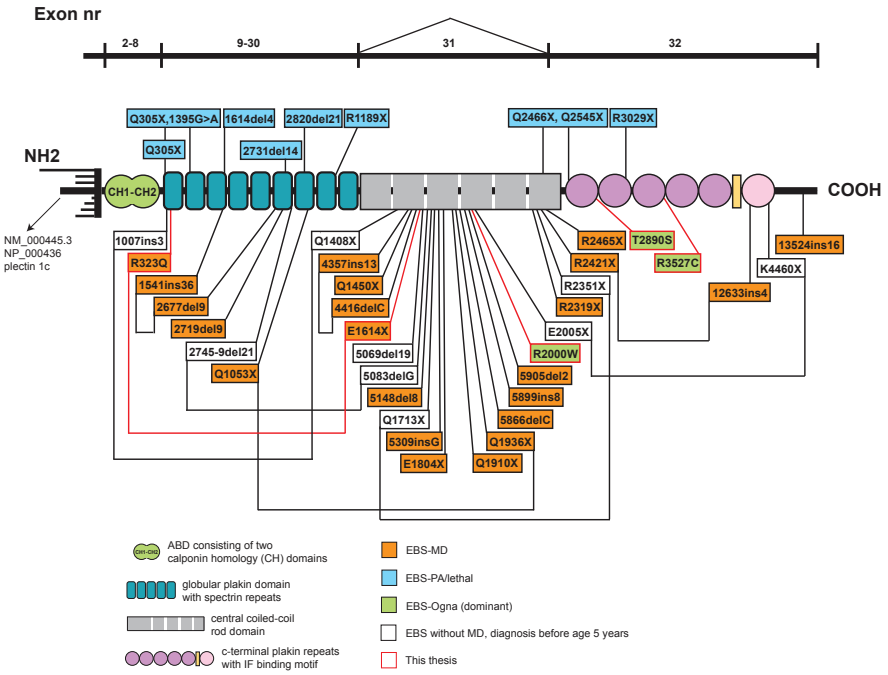

Figure 2. Plectin protein with its different domains (explained in the legend below the protein) and all previous reported *PLEC1* mutations in humans with the associated phenotypes. Sequence numbering is according to RefSeqs NM_000445.3 (mRNA) and NP_000436.2 (protein). Eight human plectin isoforms, differing in the N-terminal part preceding the actin-binding domain, are reported in GenBank. In this thesis evidence is found for the presence of an additional plectin transcript lacking the rod domain encoded by exon 31, the presence of which was already shown in animals.

Unsolved basal EBS: no *KRT5*, *KRT14* or *PLEC1* mutation

In 12 of the basal EBS probands reported in **chapter 2** and **3** no mutation was found in the genes *KRT5*, *KRT14*, or *PLEC1*. In these biopsy-proven basal EBS probands the question remains what the underlying molecular defect is. Interestingly, two probands show a typical EBS-DM phenotype, including clumping of keratin observed in electron microscopy (EM) of skin biopsies. One of these probands is part of a larger family with several affected persons from different generations. As discussed in **chapter 2**, mutations in the gDNA encoding *KRT5* and *KRT14* were excluded by regular PCR amplification and direct sequencing of exons with exon-intron

borders of *KRT5* and *KRT14* gDNA. In addition, analysis of the long-range PCR gDNA and cDNA products did not indicate larger deletions or insertions or intronic splicing-affecting mutations that might be missed with the above mentioned, regular mutation-screening. Furthermore, K5 and K14 expression was unreduced, also in the sporadic cases, excluding recessive null mutations. However, the smallest exons of *KRT5* and *KRT14* are 35bp and 47bp long. Therefore, a heterozygous in-frame deletion of one these exons might be missed by amplification of single exons and by analysis of long-range PCR product lengths as well, because the resolution of the gels is not sufficient to detect these small deletions. In addition, an intronic mutation that produces an alternative splice-site, could lead to the deletion or insertion of a short sequence in the transcript, or out-splicing of one of the small exons, both of which would be missed by analysis of long-range PCR product length. Copy number variations of the genes may have been missed as well. Additional Northern blots, Southern blots, and analysis of the products of amplifying smaller overlapping parts of cDNA, are necessary to investigate these possibilities. Whole genome linkage analysis of the EBS probands with large pedigrees is another possibility to reveal the locus of the pathogenic mutation.

Another candidate gene for involvement in EBS was *KRT15*, encoding K15, another type I keratin expressed in basal keratinocytes. However, *KRT15* mutation analysis in groups of EBS patients without *KRT5* and *KRT14* mutations from the United Kingdom and Australia/New-Zealand, was negative.[46, 62]

Other candidate genes for involvement in non-syndromic EBS are genes encoding proteins that anchor the basal keratins to desmosomes and hemidesmosomes, such as DP, BP230, BP180 and integrin $\alpha 6\beta 4$ (discussed in **chapter 2**). Liovic et al. very recently showed that mutations in the helix boundary motifs of K5 and K14 that were associated with EBS-DM caused down-regulation of multiple cell-cell and cell-matrix junctional protein-encoding genes, among which desmosomal, hemidesmosomal and gap junction genes.[63] These findings suggest a close interaction and cross-talk between the keratin cytoskeleton and the cell junctions. Mutations in the junctional proteins that anchor the keratin cytoskeleton of basal cells could possibly lead to phenotypes resembling the phenotype associated with mutations in that keratin skeleton itself.

Keratins, and IFs in general, are the substrate for several post-translational modifications that regulate function and assembly, such as phosphorylation, and transamination (reviewed in [36]). Although highly speculative, mutations in proteins involved in these regulatory functions could potentially impair keratin function. Another speculation to be mentioned is the involvement of epigenetic phenomena leading to K5 and K14 pathology. Epigenetics (meaning 'on top of genetics') refers to changes in phenotype or gene expression caused by mechanisms other then changes in underlying DNA sequence, or more specific: heritable traits that do not involve changes to the underlying DNA sequence.[64] Examples of epigenetic mechanisms are paramutation and DNA methylation. Of note, the formation of Mallory-Denk bodies, which are hepatocyte inclusion bodies containing K8 and K18 and are associated with mutations in these proteins, involve epigenetic factors.[65-67] Cytoplasmatic keratin-containing bodies ('clumping')

are also observed in EBS-DM and EI, and several other hereditary IF diseases show inclusion bodies as well (reviewed in [36]). These observations are interesting in the light of our two families with EBS-DM (and keratin clumping), in which no *KRT5* or *KRT14* mutations could be found. In general, epigenetics is a rapidly expanding research field. Future (and perhaps ongoing) RNA and protein studies, compared with results of genomic DNA analysis, will reveal the role of epigenetics in the pathogenesis of EBS and may provide treatment possibilities. Elucidation of the molecular background of EBS cases in which K5 and K14 mutations do not underlie the keratinocyte fragility may provide insight in additional proteins, and mechanisms, involved in the maintenance of basal keratinocyte integrity, and provide new ideas for therapy of EBS.

Hereditary cardiocutaneous syndromes

The cases presented in **chapter 5, 7,** and **8** can all be regarded as cardiocutaneous syndromes based on their phenotype. The cases in **chapter 7** and **8** add two new phenotypes to the group of desmosomal cardiocutaneous syndrome (DCCS), defined as such because of sharing their aetiology in mutated desmosomal proteins that are shared by skin and heart. In **chapter 6** these syndromes and their molecular background are reviewed and discussed.

A contiguous gene syndrome involving both skin and heart: the importance of array comparative genome hybridisation (aCGH) analysis in case of additional unexplainable clinical features in monogenic disorders

In **chapter 5** an exceptional patient with Kindler syndrome (KS) is described. This patient showed the full range of clinical features of KS: trauma-induced skin blistering, photosensitivity, poikiloderma, gingival fragility, ectropion, esophageal and urethral stenosis, intestinal problems, actinic keratoses and squamous cell carcinoma. Mutation analysis of the gene *FERMT1* encoding the focal adhesion protein fermitin family homologue-1 (FFH-1, also called Kindlin-1) confirmed the clinical diagnosis on a molecular level: a (seemingly) homozygous complex deletion mutation was found. This mutation caused a frameshift and a subsequent premature termination codon 14 codons downstream (p.Ser142LeufsX14), predicted to result in loss-of-expression, or a truncated *FFH-1* protein lacking its functional domains. However, additional clinical features, such as mild mental retardation, scoliosis, joint deformities of the distal extremities, and cardiac hypertrophy with ECG abnormalities were present. Considering the restricted expression of FFH-1 to epithelial tissues, these additional clinical features could not be explained by the *FERMT1* mutation.[68] An aCGH showed a monoallelic ~3 Mb microdeletion on chromosome 20p12.3 involving the complete *FERMT1* gene, and in addition several other genes among which also cardiac expressed genes and genes involved in brain and skeletal development. Thus the patient had a contiguous gene syndrome with homozygous loss-of-function of *FERMT1* leading to features of KS and additionally haploinsufficiency of other genes leading to the mental retardation, and cardiac and skeletal/joint abnormalities. A recent publication involved

five cases with heterozygous microdeletions in the same region of the genome.[69] The smallest deletion involved only the gene *BMP2* that codes for bone morphogenic protein-2 (BMP-2), and is expressed in various tissues. BMP-2 is thought to be involved in myocardial patterning, and neural, bone and cartilage development. This particular patient showed dysmorphic features, small stature, and neurocognitive delay similar to our patient, and also a cardiac arrhythmia disorder called Wolff-Parkinson-White (WPW) syndrome. ECG examination did not reveal signs for WPW in our patient, but other ECG abnormalities and a ventricular hypertrophy were clearly present. While the effect of heterozygous loss of one of the other genes in this region can not be excluded with certainty, these data suggest that hemizygosity for *BMP2* might be involved in the additional clinical features (next to the features of KS) in our patient. In case of seemingly recessive inheritance of a monogenic disease with additional clinical features that cannot be explained by the expression of the gene involved, a screening for larger genomic deletions is warranted to prevent confusion about the clinical features associated with the monogenic disease, and unnecessary anxiety for other patients with that disease. Furthermore, certainty about the precise molecular diagnosis is important for adequate counselling and a more accurate prognosis for the patient and his family.

As mentioned above, the patient showed all mucocutaneous features of KS. Although at present not all the other genes that are heterozygously deleted in our patient are well characterized concerning expression and function, the current data in the NCBI database indicate that the other deleted genes on chromosome 20p12.3, besides *FERMT1*, are not expressed in high levels in the skin. Thus the mucocutaneous clinical features likely reflect loss-of-function of FFH-1, although additional effects of heterozygous loss of expression of other genes on the skin phenotype cannot completely be excluded. The development of multiple actinic keratoses and a squamous cell carcinoma in our KS patient underscore the importance of regular examinations for mucocutaneous malignancies and the prevention of UV exposure from an early age in patients with KS.

KS is the first hereditary disorder in humans caused by a defect in a focal adhesion protein. Focal adhesions are dynamic structures that connect the actin filament system to the extracellular matrix via integrin heterodimers and multiple other proteins. Focal adhesions mediate 'outside-in' and 'inside-out' cell signalling, hereby controlling cell behaviour. FFH-1 belongs to a family of proteins to which also FFH-2 (chromosome 14q21.1, expressed in dermis, heart, skeletal muscle, lung, kidney, bladder, stomach), and FFH-3 (chromosome 11q13.1, expressed in the hematopoietic system), belong.[68, 70, 71] These proteins are thought to regulate integrin activation.[72-74] Indeed we observed abnormal expression of integrins α3, α6 and β4 in skin of our patient. In addition, several hemidesmosomal proteins and type VII collagen showed an interrupted and widened BMZ staining, indicating that loss of focal adhesion integrity affects the complete BMZ morphology. This is supported by EM findings of small and hypoplastic hemidesmosomes in the patient's skin samples. Furthermore, *in vitro* data of other studies have indicated that FFH-1 is required for integrin β1-mediated keratinocyte adhesion, keratinocyte

polarisation, proliferation, and migration.[75, 76] However, the mechanisms by which loss of FFH-1 function causes skin atrophy and skin fragility, and the reasons for the evolvement of the clinical features of KS patients during life are poorly understood. Similarly, the association of KS due to FFH-1 loss-of-function mutations with the increased risk for development of mucocutaneous malignancies remains to be elucidated. A better understanding of FFH-1 associations with other proteins and the pathways/mechanisms by which FFH-1 regulates integrin activation may provide insight in these matters. At present, positive anti-kindlin-1 immunolabeling can not rule out KS, as is also shown by the positive staining in our KS patient. Development of anti-human FFH-1-specific antibodies against different domains of the proteins may: 1) facilitate diagnosis, 2) facilitate *in-vivo* investigations of KS skin, and 3) allow studies investigating the effect of different mutations on protein expression, and subsequently the development of a comprehensive genotype-phenotype correlation for KS.

Lethal acantholytic EB as a desmosomal cardiocutaneous syndrome (DCCS)

Chapter 8 comprises the second report of lethal acantholytic epidermolysis bullosa (LAEB) and the data confirm mutations in the *DSP* gene, coding for DP, to underlie this syndrome. However, it has to be mentioned that only three cases have been reported at present. Mutation analysis in clinically similar cases will have to reveal if only *DSP* mutations cause a LAEB phenotype, or whether other genes may be involved as well. In the proband described in this thesis a markedly enlarged heart was observed. The first reported LAEB case showed cardiac dilatation too, and died of heart failure.[77] However, microscopically no signs of fibrosis or fat depositions, as can be seen in patients with dilated cardiomyopathy (DCM) and arrhythmogenic right ventricular cardiomyopathy (ARVC) due to other DP mutations, were observed.[78, 79] Therefore, the cardiomyopathy and heart failure in this first LAEB patient were considered to be a consequence of the enormous intravenous fluid supplements that were necessary to compensate for the massive transcutaneous losses.[77] However, the additional finding of intrauterine cardiac enlargement in the LAEB patient reported in **chapter 8,** suggests that cardiomyopathy might be a feature of the LAEB syndrome. Furthermore, all other *DSP* mutations (except one missense mutation, in compound heterozygous state with an N-terminal nonsense mutation, associated with skin fragility-woolly hair syndrome) affecting the DP C-terminus are associated with cardiomyopathy, although not in the neonatal period (figure 3). DP harbours multiple additional IF-interacting sites in its C-terminus in addition to the major IF-binding site between the B- and C-subdomain. We therefore speculate that the later onset of the cardiomyopathy in the other cases with mutations that only affect parts of the DP C-terminus is due to some remaining unaffected but weaker DP-IF interaction (figure 4), whereas in the first LAEB case described by Jonkman *et al.*[77] the complete C-terminus of both DP isoforms was missing. Similarly, in the LAEB cases described in this thesis, it is also very likely that a DP protein lacking its complete C-terminus (+ rod) is resulting from the mutation. The C-terminus of DP is essential for the binding of IFs in skin (keratins) and heart (desmin). Therefore, in the LAEB cases the DP-IF connection at cell-cell

contacts, that was obviously lost in skin, is likely also lost in myocardium. Presumably, in both skin and heart there is another protein capable of anchorage of IFs to the desmosomal plaque, as complete loss of keratin anchorage to the desmosome is unlikely to allow intrauterine cardiac development and survival.[80] A possible candidate for compensation of DP loss is another plakin protein, plectin, which is localized to desmosomes and is also able to bind keratins in skin and desmin in heart.[81-83]

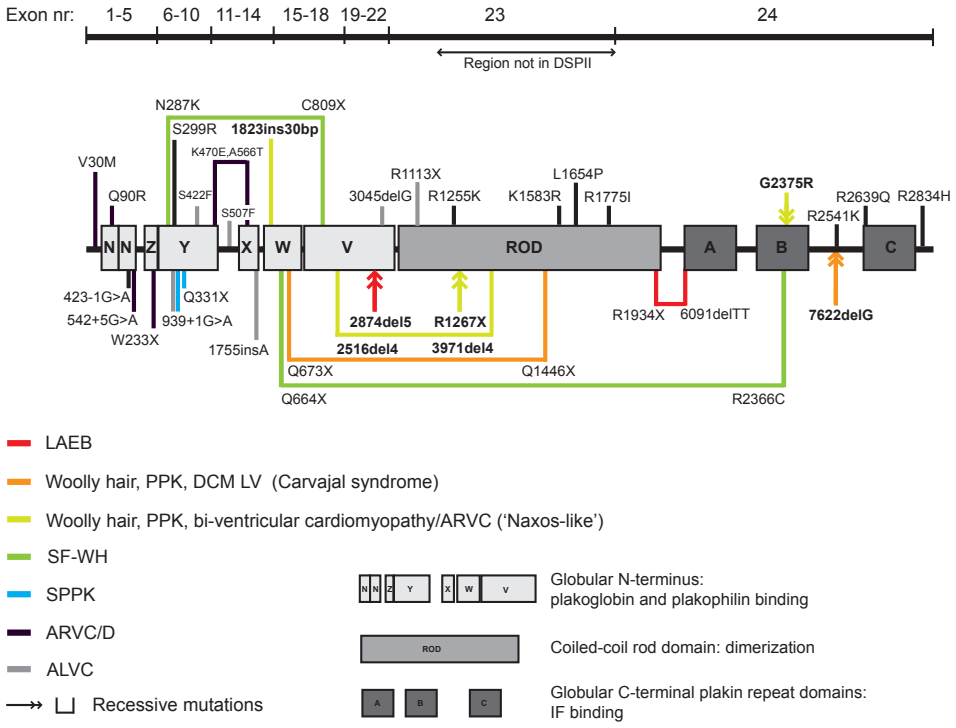

Figure 3. Schematic representation of the DP protein with all previous reported *DSP* mutations and the mutation found in this thesis (chapter 8). GenBank accession no. NM004415.2 (mRNA) and NP004406.2 (DP I isoform). ARVC/D, arrhythmogenic right ventricle cardiomyopathy/dysplasia; ALVC/D, arrhythmogenic left ventricular cardiomyopathy/dysplasia; DCM, dilated cardiomyopathy; LAEB, lethal acantholytic epidermolysis bullosa; LV, left ventricle; PPK, palmoplantar keratoderma; SF-WH, skin-fragility woolly-hair syndrome; SPPK, striate palmoplantar keratoderma.

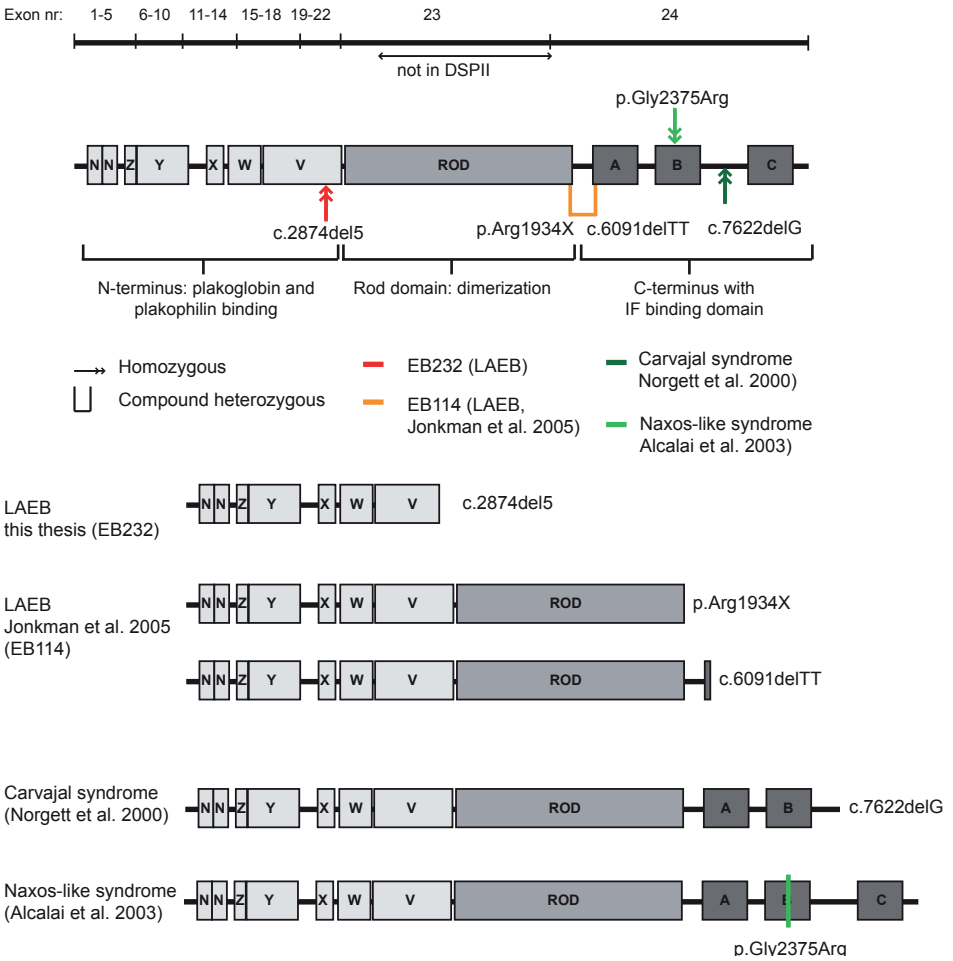

Figure 4. Schematic representation of the DP protein with the reported recessive *DSP* mutations affecting the C-terminus (above) and the LAEB mutation reported in chapter 8. The resulting (predicted) DP proteins from each of these mutations are depicted below.[77, 84, 85]

Plectin defects in human cardiac disease

Plectin is a rather obscure desmosomal protein which is not considered in the majority of articles reviewing desmosomes. Plectin is expressed in a multitude of tissues where it is mainly localized at connection structures, like intercalated discs (IDs) and Z-discs in myocardium; hemidesmosomes, desmosomes and focal contacts in skin; Z-discs and costameres in skeletal muscle; desmosomes in intestinal epithelium, etc.[61, 83, 86-89] Plectin belongs to the family of plakin proteins to which also desmoplakin and BP230 belong, and functions as a major cytolinker interconnecting the three major cytoskeletons (actin, microtubules and IFs) and linking them to the above mentioned cell-cell and cell-matrix structures. In the desmosome plectin was

shown to colocalize with DP.[83] However, the function of plectin in the desmosome seems to be 'accessory' as neither of the reported plectin mutations caused desmosomal disintegration and/or acantholysis. Whatever its function in the desmosome may be, plectin-knockout mice survive until birth, but die in the first postnatal days revealing cardiac abnormalities in addition to considerable skin fragility and skeletal muscle pathology.[61] Furthermore, specific skeletal and cardiac muscle plectin-knockout mice showed (exercise-induced) cardiac pathology as well.[90] These observations made it tempting to speculate that plectin mutations may be involved in cardiocutaneous syndromes and non-syndromic cardiomyopathies, like DCM and ARVC, as well, similar to the other plakin protein desmoplakin (discussed in the previous paragraph). In **chapter 7** a patient with a cardiocutaneous syndrome due to plectin mutations is presented. The patient suffered from EBS with adult-onset muscular dystrophy (MD) and DCM. Myocardial tissue studies revealed fibrosis and an altered desmin skeleton. The patient was compound heterozygous for a previously reported *PLEC1* nonsense mutation and a missense mutation in the plectin N-terminus. We speculate that the N-terminal mutation interfered with plectin incorporation into desmin-anchoring structures, like Z-discs, and desmosomes in the ID, with subsequent impaired desmin organisation. Interestingly, two previously reported Dutch patients with recessive EBS with MD (EBS-MD) due to a homozygous nonsense *PLEC1* mutation, Q1804X, had a cardiomyopathy as well (not mentioned in the publication).[91] These patients both died of respiratory failure. The cardiomyopathy became relevant again when a related and potential heterozygous carrier of the mutation suddenly died of acute heart failure and turned out to have a severe DCM. In addition, his father suffered sudden death in his early forties. This large family is currently under investigation to see whether the cardiac disease in this family segregates with the plectin mutation. Furthermore, cardiomyopathy has been mentioned in another patient with plectin mutations.[59] Currently, *PLEC1* DNA mutation analysis is performed in a group of probands with ARVC or ARVC-like disease, in which mutations in several desmosomal proteins have been excluded. It would be interesting to do the same in hereditary DCM. The results will reveal the possible role of plectin mutations in these diseases.

Clinical features of DCCS: beyond cell-cell adhesion defects

In **chapter 6** the DCCS are discussed, of which Carvajal syndrome due to a recessive DP C-terminal truncating mutation, and Naxos disease due to a recessive plakoglobin (PG) C-terminal truncation, are the most well known.[85, 92, 93] One of the current topics in DCCS, and disease resulting from mutations in desmosomal proteins in general, is to what extent cell-signalling pathways are involved in the pathogenesis. Recent studies implicate functions of desmosomal proteins that go beyond cell-cell adhesion and involve morphogenesis and cell signalling pathways, such as the canonical WNT/β-catenin signalling pathway.[80, 94-97] It is thought that under physiological conditions a delicate balance exists between the amount of desmosomal proteins and adherens junction-proteins at cell-cell borders and the amount in the cytoplasm, which can potentially translocate to the nucleus and influence gene expression.

The Wnt signalling pathway is involved in regulating degradation of PG and keeping the concentration low in the cytoplasm.[98] Activation of the Wnt pathway leads to intracellular PG accumulation, increased nuclear transport and changes in gene expression.[99] Possibly mutated desmosomal proteins perturb the balance between cytoplasmatic and junctional amounts and subsequently influence the Wnt signalling pathway and nuclear gene expression. Several studies have shown cytoplasmatic accumulation of desmosomal proteins in case of mutations in one of the desmosomal proteins.[78, 80, 100-102] Not only is the mutated desmosomal protein affected, but the localisation of other desmosomal proteins as well. The clinical features observed in DCCS, such as woolly hair, palmoplantar keratoderma, hyperkeratosis, and cardiomyopathy may thus be the reflection of altered signalling pathways and changes in gene expression, and not merely of impaired cell-cell contact.

Interesting is the observation that not only desmosomal protein expression is affected in case of desmosomal protein mutations, but expression of other junctional proteins as well. In the cardiocutaneous syndromes Carvajal syndrome and Naxos disease due to recessive mutations in DP and PG, respectively, one of the early pathological alterations is the remodelling of gap junctions and the markedly diminished expression of the gap junction protein connexin 43 (Cx43) at cell-cell junctions in myocardium.[78, 102] This was also observed in ARVC due to dominant mutations in other desmosomal proteins.[101] These changes occur early in disease, even before significant structural changes have developed. Furthermore, in case of PG-knockout cardiomyocytes, and in cardiomyocytes with truncated PG, β-catenin was observed to locate at desmosome-like structures. Franke et al. have shown with immuno-EM analysis of normal murine cardiomyocytes that desmosomal proteins are expressed in both desmosome-like structures and adherens junction-resembling structures at the ID.[103, 104] Other studies also indicated interconnections between desmosomal and adherens junction proteins, like for example between α-catenin (adherens junction) and the plakophilins (desmosome).[105] All these data implicate a close interaction and interdependence between intercellular connecting structures in myocardium. In skin the localisation of desmosomal proteins and adherens junction proteins appears to be more restricted, with only PG found in both junctions. But also in skin, adherens junctions and desmosomes are dependent on each other in establishing normal cell-cell contact.[106] It would be interesting to investigate the Cx43 expression and gap junction morphology in skin of conditional PG-knockout and DP-knockout mice, and in addition in palmoplantar skin of patients with DCCS and patients with non-syndromic PPK due to mutations in DP and desmoglein-1, as both mutations in Cx43 and mutations in the desmosomal proteins DP, PG, desmoglein-1 and desmocollin-2 are associated with palmoplantar keratoderma.[85, 93, 107-111] Perhaps the pathogenesis of palmoplantar keratoderma in DCCS and non-syndromic palmoplantar keratoderma due to *DSP* mutations involves altered Cx43 and/or gap junction functioning. The earlier mentioned study of Liovic et al. revealed down-regulation of Cx43 in case of K5 and K14 mutations associated with the severe EBS-DM phenotype.[63] In this light it is interesting to note that most EBS-DM patients have palmoplantar keratoderma as well.

Alternatively, altered nuclear signalling by nuclear translocation of PG upon changes in cell-cell junction composition, and subsequent altered gene expression might cause the gap junction changes, as well as the altered proliferation and differentiation in palmoplantar skin. Similarly, the woolly hair in skin-fragility-woolly-hair syndrome, Carvajal syndrome and Naxos disease due to mutations in DP and PG might reflect altered nuclear signalling and gene expression, with subsequent changes in hair follicle morphogenesis, instead of, or in addition to, impaired cell-cell adhesion. This is supported by the observation of the absent/minimal skin fragility in Naxos disease due to PG mutations, indicating that this mutation does not severely impair cell-cell adhesion, but more likely affects other functions of this protein. Several observations, like: 1) non-syndromic hair abnormalities with similarities to the woolly hair in Carvajal syndrome and Naxos disease, can be caused by mutations in genes involved in the lipid pathways, *LIPH*[112-114] and *P2RY5*[115-117], 2) the fat depositions in cardiac samples of ARVC patients with mutations in desmosomal proteins, and 3) the upregulation of Wnt signalling and adipogenesis in myocardium of DP haploinsufficient mice[80], may point to central roles for the adipogenic/lipid pathways and WNT/β-catenin signalling changes in the pathogenesis of clinical features like woolly hair, and of ARVC as well. Studies investigating the role of the Wnt/β-catenin signalling pathway in the regulation of adipogenic/lipid-pathway genes, such as *LIPH* and *P2RY5*, may provide further insight in the mechanisms underlying the combination of clinical features observed in DCCS.

The genotype-phenotype correlation of mutations in desmosomal proteins, like DP, is complex and incompletely understood (see also figure 3). For example, why do heterozygous nonsense mutations predicted to cause loss of protein expression of one and the same protein, DP, cause such different phenotype as non-syndromic striate PPK and ARVC? More detailed RNA and protein studies are necessary to broaden the insight in the 'black box' between mutations in desmosomal genes and clinical outcome. The role of modifying polymorphisms in related proteins and the possibility of a bigenic disorder remain to be investigated. In addition, cases showing cardiocutaneous phenotypes that resemble Carvajal and Naxos syndrome have been described, in which mutation analysis of *DSP* and *JUP* did not reveal any mutation.[118, 119] Recently, a recessive mutation in *DSC2* encoding desmocollin-2 was shown to underlie a Naxos-like syndrome, and in **chapter 7** a novel DCCS due to *PLEC1* mutations is described. These data point to a broader genetic heterogeneity for these syndromes.[120] Cardiocutaneous desmosomal proteins that have not been related to cardiocutaneous syndrome at present are plakophilin-2 and desmoglein-2. In humans, autosomal dominant mutations in the genes *PKP2* (chromosome 18q12) and *DSG2* (12p11) encoding these proteins cause the cardiac phenotype of ARVC.[121-125] However, the levels of these proteins in the skin are very low, so the question remains whether mutations in these proteins will cause a skin phenotype. Plakophilin-2 -/- mice showed embryonic lethality at midgestation and desmoglein-2 -/- mice did not survive the embryonic blastocyst state, indicating an important function for these protein in early embryogenesis.[126, 127] Early lethality of the mice prohibited observation of effects of plakophilin-2 absence and

desmoglein-2 absence in the stratified epidermis. Conditional skin-knockout animals for these proteins are necessary to reveal the functions of these proteins in the skin, and more specifically: whether defects cause abnormalities.

Concluding remarks

In terms of evidence based medicine most weight is attributed to randomised controlled trials. However, the careful observation and study on single cases may also provide a wealth of insight, hypotheses and ideas of how our body functions and what to focus future studies on. I believe that their importance is exemplified by several studies in this thesis.

A wide variety of hereditary skin fragility disorders is investigated, some of which are associated with cardiac disease, with their etiology residing in mutations in proteins from various keratinocyte junctions and the keratinocyte keratin cytoskeleton. The results suggest the gene *PLEC1*, encoding the versatile and widely expressed cytolinker plectin, as an important causal gene in EBS, but also in cardiomyopathy. Future cardiac evaluation of patients with plectin mutations is indicated and these results, together with *PLEC1* mutation analysis studies in patients with idiopathic non-syndromic DCM and ARVC, will refine the role of *PLEC1* mutations in hereditary cardiomyopathy. Furthermore, *PLEC1* mutation analysis in other non-keratin-gene basal EBS populations will further establish the precise role of plectin in non-syndromic basal EBS. Studies investigating the causes of the phenotypic variability in keratin disorders, and the mechanisms behind the external and internal influences on blister severity, such as external temperature and fever, as well as inflammation, will provide ideas for development of treatment alternatives. The functional redundancy of the keratins will perhaps provide other treatment possibilities. In the hereditary desmosomal cardiomyopathies future studies investigating the role of signalling pathways, such as the Wnt/β-catenin pathway, will provide insight in pathogenesis and new modes of therapy. Results may be extended to the skin. Looking over the borders of ones own discipline, like genodermatoses and cardiogenetics in this thesis, will greatly improve insight in the fields involved.

Future issues

The future issues concerning the topics of this thesis are plentiful (see table 2). Many of them deserve further investigation. But in order to remain practical: what would be the next research project(s) if I would write grants?

The EBS patients in our cohort in Groningen who did not carry mutations in either of the genes *KRT5*, *KRT14*, or *PLEC1*, but did show a basal intraepidermal level of skin fragility deserves further analysis for the underlying molecular defect. Several approaches may be applied. The first would be to perform linkage analysis in the families with multiple affected and unaffected persons available over several generations to see whether a locus for the

pathogenic mutation can be identified. Subsequently, the candidate genes within this locus can be screened. Another possibility is to identify a candidate gene based on expression pattern and function of the encoded protein. For example, mutation screening of the exon that is specific for the epidermal isoform of BP230 is simple and quickly realizable. BP230 is homologous to plectin and expressed at the cytoplasmatic site of the hemidesmosomes where it also anchors the basal epidermal keratins to the hemidesmosomal plaque. The gene *DST* encodes several different isoforms expressed in multiple tissues, among which skin. Mutations in epidermal specific domains could possibly lead to skin fragility genodermatoses with a level of skin fragility through the base of basal keratinocytes: EBS.

Nonsense and frameshift mutations affecting K14 are associated with the autosomal dominant genodermatoses Naegelli-Franceschetti-Jadassohn-Syndrome (NFJS) and Dermatopathia Pigmentosa Reticularis (DPR). These mutations are predicted to elicit nonsense mediated RNA decay of the transcripts and this loss of K14 expression from the mutated allele. This means that these disorders are caused by K14 haploinsufficiency. However, carriers of *KRT14* mutations that in homozygous state cause recessive EBS do not show skin, hair and/or dental abnormalities. These recessive mutations also comprise mostly nonsense and frameshift mutations. There seems to be a difference between healthy carriers of recessive nonsense/frameshift *KRT14*-mutations and patients with NFJS or DPR and dominant nonsense/frameshift *KRT14*-mutations. It would be interesting to investigate the effects of the different mutations, both recessive and dominant nonsense and frameshift mutations, on the RNA transcripts and K14 protein expression, both qualitative and quantitative. Knowledge in this matter is particularly important as gene therapies are under development aiming at knocking down the mutated allele.

In chapter 4 of this thesis we have shown that a phenotype of peeling skin with palmoplantar keratoderma is associated with a *KRT1* linker domain mutation. In addition, recently we found a *KRT10* linker domain mutation in a mother and a son initially suspected from acral peeling skin syndrome (APSS, caused by recessive missense mutations in *TGM5*[128, 129]), although APSS is inherited in a recessive manner. A next research project could focus on screening patients with dyshidrosis lamellose sicca and patients with dominantly inherited peeling skin (with and without palmoplantar keratoderma) for mutations in *KRT1* and *KRT10*. The hypothesis would be that several patients in the above mentioned group carry a mutation in one of the linker domains of K1 (with palmoplantar keratoderma) or K10 (without palmoplantar keratoderma).

Currently our research group of the department of Dermatology (Groningen) is collaborating with researchers from the departments of Genetics, Cardiology and Pathology in Groningen to screen a group of patients with arrhythmogenic right ventricular cardiomyopathy (ARVC) mutations for mutations in *PLEC1*. Mutation screening of other ARVC associated genes was negative in these patients. It would be interesting to also perform *PLEC1* mutation analysis in patients with idiopathic dilated cardiomyopathy (DCM) at an early age, as dominant mutations in desmin, the intermediate filament in muscle tissue that needs plectin as an organiser cause

DCM as well. In addition, mutations in desmoplakin, another homologue of plectin, have been associated with DCM as well.

Pathogenecity of missense mutations is difficult to proof. The research group of Wiche et al. in Vienna (Austria) developed a mouse model for the EBS-Ogna plectin rod domain missense mutation p.Arg2000Trp. It would be interesting to have a mouse model for missense mutations in the N-terminus of plectin as well, to see whether these are associated with cardiac disease.

Above mentioned possible future studies are all directed towards pathogenesis/diagnostics. Currently, causative treatments (gene/RNA/protein therapy) for hereditary diseases are under development, and the lion part of attention in the genodermatoses research field is now directed in that way. However, in order to develop and to be able to implement treatments aiming at the cause of a genetic disease, knowing the underlying molecular defect is essential. Many cases remain to be solved yet, and still many lessons remain to be learned from genotype-phenotype correlations and the 'black boxes' between the genomic DNA mutation, the effect on the encoding protein, the tissue(s) involved and eventually the resulting clinical features. Therefore I plead for maintaining an at least as important role for research concerning pathogenesis/diagnostics. Our department of Dermatology at the University Medical Center Groningen is the expertise center for hereditary (and also acquired) skin fragility disorders. All patients presenting with a (skin fragility) genodermatosis are carefully documented and photographed. After receiving informed consent from the patient, DNA is obtained and fresh frozen skin samples of perilesional and healthy appearing skin are taken (in some cases additional skin biopsies for culturing are taken for further RNA/protein studies) for diagnostics, and the remains are stored for future research. Every patient has been seen by one and the same expert (prof. Dr. M.F. Jonkman), rendering a very uniform database. Altogether this provides a wealth of data and tissue for further research. In a significant number of the patients in our database, their genodermatosis cannot be explained by the current knowledge, and the molecular defect underlying their disease remains to be elucidated. Furthermore, still patients/families with novel, unsolved phenotypes appear… A challenge for the future!

Table 2. Future issues	
1	Why do *DSP* mutations, predicted to have the same effects on the desmoplakin protein, cause non-syndromic striate palmoplantar keratoderma in one kindred and non-syndromic ARVC in another kindred? Is the predicted effect on the protein not what actually happens *in vivo*? Do epigenetic factors and/or polymorphisms in other (desmosomal) proteins play a role?
2	Why do some mutations in desmoplakin (and in other desmosomal proteins) have a more deleterious effect on the right side of the heart, while others affect mainly the left side of the heart?
3	Why do some *DSP* mutations affect myocardium while others do not? Will it be possible to develop concepts as to whether *DSP* mutations will be associated with cardiomyopathy later in life or not?
4	Will the molecular defect of LAEB proof to be a desmoplakin truncation, or are other molecular defects also capable of causing this phenotype?
5	Does desmoplakin C-terminal truncation always result in cardiac pathology?
6	Are *DSG2* and *PKP2* mutations also involved in cardiocutaneous syndromes? Do mutations in these proteins cause a skin phenotype?
7	What are the mechanisms behind the development of woolly hair and palmoplantar keratoderma in patients with mutations in the desmosomal proteins desmoplakin, desmoglein-1 and plakoglobin? Are they merely the result of impaired cell-cell contact and compensatory mechanisms? Or do they reflect changes in cell signalling pathways, i.e. the Wnt/β-catenin pathway? Do mutations in desmosomal proteins influence other intercellular junctions and their proteins, like adherens junction and gap junctions, and hereby exert an indirect effect on hair follicle development and skin proliferation and differentiation?
8	Is fibrofatty displacement of cardiomyocytes always mediated by plakoglobin nuclear translocation (and upregulation of adipogenic transcription factors)?
9	Will ARVC turn out to be a disease of the WNT/β-catenin signalling pathway, instead of a desmosomal disease? And will this offer a 'final common pathway' where therapies can be aimed at?
10	What is the position and function of plectin in desmosomes in epidermis and myocardium?
11	What are the exact tissue and cell-structure expression patterns and functions of the many different isoforms of plectin?
12	Are *PLEC1* mutations also involved in hereditary non-syndromic ARVC and/or DCM?
13	What is the underlying molecular defect in the EBS patients/families in which mutations in *KRT5*, *KRT14*, and *PLEC1* have been excluded? Do epigenetic phenomena play a causative role? Or mutations in proteins involved in post-translation modifications of the basal keratins?
14	How do mutations in *FERMT1* cause the atrophic changes in the skin, the photosensitivity, and the increased risk for development of squamous cell carcinomas?
15	Why are heterozygous carriers of recessive KRT14 loss-of function mutations healthy, whereas patients with the autosomal dominant genodermatoses NFJS and DPR also carry heterozygous nonsense and frameshift mutation in KRT14 that are thought to cause loss of protein expression from the mutated allele? In other words: does haploinsufficiency for keratin 5 and keratin 14 and for keratins in general exist?
16	What mechanisms underlie the improvement of skin blistering during life, during feverish illness, and in cold and dry environments in EBS patients?
17	Will the functional redundancy of keratins proof to be useful for therapeutic approaches?
18	What are the functions of the variable head and tail domains of keratins?
19	Can we identify genetic modifiers in EBS?
20	Are K10 linker domain missense mutations associated with a peeling skin-like phenotype without palmoplantar keratoderma?
21	What is the role of epidermal keratins in TNF-α regulation? And subsequently, could TNF-α antagonists improve skin blistering in severe EBS-DM?

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Table 1. All *PLEC1* mutations in humans reported up to October 1st, 2009.

Mutation 1[$]	Exon	In frame /FS/PTC	Mutation 2[$]	Exon	In frame/ FS/PTC	Pheno-type	Onset MD (y)	Age of diagnosis	Additional features	Reference
Arg323Gln	9	Missense	Glu1614X	31	PTC	MD	***33***	40	DCM	This thesis
1541ins36	15	In frame	2677del9	15	In frame	MD	42	48	Nail dystrophy	130
2717del9	21	In frame	2717del9	21	In frame	MD	30-35	46	Nail dystrophy	130-132
Gln1053X	25	PTC	Gln1936X	31	PTC	MD	infancy	9	Tooth decay, nail dystrophy, urethral strictures	132, 133
4357ins13	31	FS > PTC	4416delC	31	FS > PTC	MD	20	40	Nail dystrophy; loose teeth; laryngeal web at birth; mucosal involvement with supraglottic stenosis and urethral strictures	134
Gln1450X	31	PTC	Gln1450X	31	PTC	MD	19	49	no	54
Glu1614X	31	PTC	E1614X	31	PTC	MD	10-20	no description phenotype	no description phenotype	130
5146del8	31	FS > PTC	5146del8	31	FS > PTC	MD	10	24	Nail dystrophy, teeth abnormalities	135
5309insG	31	FS > PTC	5309insG	31	FS > PTC	MD	1	no description phenotype	no description phenotype	130
Glu1804X	31	PTC	Glu1804X	31	PTC	MD	15	46	Mental retardation and epilepsy (LV-cardiomyopathy*)	91
Gln1910X	31	PTC	Gln1910X	31	PTC	MD	infancy	29	PPK, nail dystrophy	136, 137
5864delC	31	FS > PTC	5864delC	31	FS > PTC	MD	~30	45	Nail dystrophy; urethral strictures	131, 132, 138-140
5897ins8	31	FS > PTC	5897ins8	31	FS > PTC	MD	<2	12	no	141
5903del2	31	FS > PTC	5903del2	31	FS > PTC	MD	<10	10	Dystrophic nails, focal PPK, severe mucosal involvement, supraglottic stenosis	139, 142
Arg2319X	31	PTC	Arg2319X	31	PTC	MD	25	52	no	143
Arg2421X	31	PTC	12631ins4	32	FS > PTC	MD	5	33	Nail dystrophy, partial scarring alopecia	132, 133
Arg2465X	31	PTC	Arg2465X	31	PTC	MD	<2	33	Severe mucosal involvement with supraglottic stenosis; brain atrophy	139, 141
13523ins16	32	FS > PTC	13523ins16	32	FS > PTC	MD	11	25	Mild LVH; brain atrophy+hydrocephalus; cataract; arteriovenous malformation	59

(Table 1. Continued)

Mutation 1[$]	Exon	In frame /FS/PTC	Mutation 2[$]	Exon	In frame/ FS/PTC	Pheno-type	Onset MD (y)	Age of diagnosis	Additional features	Reference
1005ins3	9	In frame	Gln1408X	31	PTC	EBS	not present	4	no	[144]
2743-9del21	i22-e23	Splice site mutation	5081delG	31	FS > PTC	EBS (MD?)	not present	< 1 y	no	[145]
5067del19	31	FS > PTC	5067del19	31	FS > PTC	EBS	not present	5	Nail dystrophy, mild focal PPK, severe mucosal involvement; supraglottic stenosis	[142]
Gln1713X	31	PTC	Arg2351X	31	PTC	EBS	not present	4	Severe mucosal involvement; nail dystrophy; supraglottic stenosis	[146]
5307insG	31	FS > PTC	5307insG	31	FS > PTC	EBS	not present	3	Nail dystrophy, severe mucosal involvement with supraglottic stenosis	[147]
Glu2005X	31	PTC	Lys4460X	32	PTC	EBS	not present	< 1	Cleft lip and palate	[145]
Arg2000Trp	31	Missense	n.a.	n.a.	n.a.	Ogna/ plectin-EBS	not present	>50	Onychogryphosis	[50,52] and this thesis
Arg1853Trp	31	Missense	n.a.	n.a.	n.a.	Ogna/ plectin-EBS	not present	18	Nail abnormalities	This thesis
Thr2890Ser	32	Missense	n.a.	n.a.	n.a.	Ogna/ plectin-EBS	not present	>40	Nail abnormalities	This thesis
Arg3527Cys + Thr3534Met	32	Missense	n.a.	n.a.	n.a.	Ogna/ plectin-EBS	not present	>50	Nail abnormalities	This thesis
Gln305X	9	PTC	Q305X	9	PTC	PA	not present	neon lethal	Ear deformities, aplasia cutis	[148]
Gln305X	9	PTC	1393G>A	12	splice site mutation	PA	not present	1	Aplasia cutis	[149]
1612del4	15	FS > PTC	1612del4	15	FS > PTC	PA	not present	neon lethal	no	[148]

(Table 1. Continued)

Mutation 1[$]	Exon	In frame /FS/PTC	Mutation 2[$]	Exon	In frame/ FS/PTC	Pheno-type	Onset MD (y)	Age of diagnosis	Additional features	Reference
2729del14	22	FS > PTC	2729del14	22	FS > PTC	PA	not present	neon lethal	Dismorphias; brain atrophy; extensive aplasia cutis	[150]
2818del21	23	In frame	2818del21	23	In frame	PA	not present	neon lethal	Aplasia cutis	[148]
Arg1189X	27	PTC	Gln2538X	32	PTC	PA	not present	neon lethal	Aplasia cutis	[149]
Gln2466X	31	PTC	Gln2545X	32	PTC	PA	not present	4 months	no	[54]
Arg3029X	32	PTC	Arg3029X	32	PTC	PA	not present	neon lethal	no	[148]

DCM, dilated cardiomyopathy; EBS, epidermolysis bullosa simplex; FS, frameshift; IF, immunofluorescence; LVH, left ventricle hypertrophy; MD, muscular dystrophy; n.m., not mentioned; PA, pyloric atresia; PPK, palmoplantar keratoderma; PTC, premature termination codon; y, year

*Not reported in the published article.

[$]GenBank accession no.: NM000445.3 (mRNA) and NP000436.2 (plectin isoform 1c), numbering starting at first nucleotide.